used: 1. Because the recovery after thawing is less than 1%, large numbers of L_3's must be frozen (>100,000). 2. Upon thawing, the larvae should be incubated in BU at room temperature for 20 to 24 hr, allowing the injured L_3's to die. Thereafter, the living L_3's are collected. 3. Although it is not absolutely necessary, immunosuppression of the rat, before and after the subcutaneous injection of the L_3's, can be used to increase the number of adults maturing in the small intestine. 4. Because few adults develop from cryopreserved L_3's, it is necessary to amplify the infection by passage through another rat to obtain sufficient *S. ratti* for most experimental purposes.

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Research Note

Histochemical Observations on *Cyathostoma lari* (Strongyloidea: Syngamidae)

W. THRELFALL
Department of Biology, Memorial University, St. John's, Newfoundland, Canada A1B 3X9

ABSTRACT: The location and relative abundance of chemical components of adult female *Cyathostoma lari* were determined using a variety of histochemical methods. Carbohydrates, acid mucopolysaccharides, lipids, and proteins were widely distributed throughout the nematode. Host blood and hemoglobin were detected in the gut lumen, with hemoglobin also being demonstrated in the pseudocoel. Ribonucleic acid, mitochondria, succinic dehydrogenase, and acid and alkaline phosphatases were located in the digestive and reproductive systems. The data suggest that the anterior intestine plays the most important role in digestion. The role and importance of the various substances are discussed.

KEY WORDS: histochemistry, Nematoda, *Cyathostoma lari*.

Much work has been performed on the physiology and biochemistry of metazoan parasites, particularly digeneans and cestodes (von Brand, 1979; Chappell, 1980; Barrett, 1981; Smyth and McManus, 1989). One of the least examined groups has been the nematodes, with species of medical and veterinary importance receiving the most attention. Within this group larvae and eggs have been the objects of greatest attention. In examining the chemical constituents of nematodes, studies have to a large extent been performed using homogenates of whole worms (Singh and Sharma, 1981; Chopra, 1986; Rao and Rajlingam, 1989). Few studies have been concerned with determining the precise location of various substances within nematodes (Sood and Kalra, 1977; Sood and Sehajpal, 1978; Maki and Yanagisawa, 1980; Sharma and Singh, 1985).

The distribution of the chemical components of a parasite is a reflection of where biochemical processes are occurring, with intensity and type of reaction perhaps changing at different times during the host's life cycle. Host physiology and location of the parasite within the host will also affect the physiological status of the parasite. Phylogenetic differences among hosts and among parasite species might also be reflected in the parasite's physiology. As noted by Threlfall et al.

(1990), data to support such a contention are rare. The present histochemical study was undertaken to determine the distribution and relative concentrations of various substances throughout the body of adult female *Cyathostoma lari* Blanchard, 1849, recovered from herring gull (*Larus argentatus* Pontoppidan, 1763) chicks taken in both Wales and Newfoundland, and to compare the results with previous works. *Cyathostoma lari* is an obligate, blood-sucking nematode that lives in the nasal cavities of a variety of birds including gulls (*Larus* spp.) (Barus et al., 1978). A plug of host mucosa is drawn into the buccal cavity of the worm and lacerated with cutting plates so that blood may flow freely through the worm (Threlfall, 1966; Colam, 1971a). This study also expands on the work of Colam (1971a) who studied the gut ultrastructure and digestive physiology of this species, as well as *Rhabdias bufonis, R. sphaerocephala,* and *Cosmocerca ornata* (Colam, 1971b, c).

Worms were fixed in situ by flooding the sinuses of freshly killed gull chicks with a variety of fixatives, including Altmann's, cold acetone (4°C), 90% ethyl alcohol, Susa's, and Zenker's depending on the tests to be performed. The worms were then removed from the sinuses, blocked at 54°C in paraffin wax, and sectioned at 5, 10, and 15 μm. A small number of worms were recovered alive, quick-frozen, and sectioned at 10 and 15 μm using a freezing microtome. Carbohydrates were detected in Zenker's-fixed worms, using Best's carmine, and the PAS reaction, with and without amylase treatment (Gurr, 1956, 1958; Pearse, 1968). The presence of acid mucopolysaccharide was revealed in Susa's-fixed specimens using the thionin, toluidine blue, and Hale's dialysed iron methods outlined by Gurr (1958). Lipids were stained using Sudan Black B in specimens fixed in Altmann's and Zenker's or in frozen specimens, or copper phthalocyanin using methanol fast blue stain (Pearse, 1968). Controls were with Sudan Black B plus pyridine extraction. Mercuric bromphenol blue with and without deaminase was used to detect proteins in Zenker's-fixed worms (Mazia et al., 1953; Pearse, 1968). The digestive tract of the nematode contained host blood, and the pseudocoel was filled with a red pigment-containing fluid. To determine the distribution of hemoglobin within the worm, Zenker's-fixed sections were stained with Giemsa stain and benzidine (Gurr, 1958). Ribonucleic acid was detected using the pyronine/methyl green method with and without ribonuclease (Gurr, 1958). Mitochondria were demonstrated in frozen sections by the presence of succinic dehydrogenase using the NBT technique (Pearse, 1972; Bancroft, 1975) and in Altmann's-fixed sections with Metzner's stain (Metzner and Krause, 1928). Acid phosphatase was detected using the Gomori lead nitrate, Burnstone's, and the modified Gomori methods (Pearse, 1968), whereas alkaline phosphatase was demonstrated using the calcium cobalt (Gomori, 1952) and modified Gomori methods (Pearse, 1968). Tests for phosphatases were performed on material that had been fixed in cold acetone (4°C), except in the case of the modified Gomori test for alkaline phosphatase where specimens were fixed in 90% ethanol. Controls were performed as follows: acid phosphatase, incubated as for the test but 0.01 M sodium fluoride was included in the reaction medium; alkaline phosphatase, 3% sodium-b-glycerophosphate in the medium was replaced by distilled water (Chayen et al., 1973).

The distribution and amounts or activity of various chemical components in the digestive and reproductive tracts of *C. lari* are shown in Table 1. Glycogen comprises the principal carbohydrate reserve in nematodes, with the amount varying from species to species (Fairbairn, 1958, 1960; Barrett, 1981). A general orthochromasia was noted throughout the worm with the highest concentrations of carbohydrates being seen in the anterior intestine (Table 1). Sood and Sehajpal (1978) noted similar results in *Haemonchus contortus.* The ovaries of *C. lari* showed a similarly intense reaction. The cuticle of this helminth is extremely thin, particularly when compared to that of many intestinal inhabitants, e.g., *Ascaris lumbricoides,* and was negative in tests for carbohydrates, whereas the hypodermis and cytoplasmic portion of the muscle cells stained lightly. A similar situation was noted by Sood and Kalra (1977) in *Haemonchus contortus* and *Xiphinema insigne.* Acid mucopolysaccharide, an ubiquitous substance in nematodes (Barrett, 1981), was present in all the body tissues except the cuticle and the eggshell/membranes. Sood and Kalra (1977) noted the presence of this substance in the inner cortical layer of the cuticle of *H. contortus* and *X. insigne.* Sood and Sehajpal (1978) were unable to demonstrate acid mucopolysaccharide or glycogen in the brush border of the gastrodermis of *H. contortus.* In the present study, considerable amounts of both glycogen and acid mucopolysaccharide were present in the

Table 1. Histochemistry of the digestive and reproductive systems of adult female *Cyathostoma lari*.

	Body region								
	Buccal region	Esoph-agus	Intestine			Rectum	Ovary	Ovum (in shell)	Egg-shell
			Ant.	Mid.	Post.				
Carbohydrates	+	+	++	+	+	+	++	+	−
Acid mucopolysaccharide	+	+	+	+	+	+	+	+	−
Lipids	−	−	+++	++	++	−	++	+	++
Proteins	+	+	++	++	+	+	+++	++	+
Ribonucleic acid	−	−	+++	++	++	+	+++	+	−
Mitochondria	−	−	+++	++	++	+	++	+	−
Succinic dehydrogenase	−	−	+++	++	++	+	++	+	−
Acid phosphatase	−	−	+++	++	+	+	++	+++	−
Alkaline phosphatase	−	−	+++	++	+	+	++	+++	−

+++ = strongly positive; ++ = moderately positive; + = weakly positive; − = negative.

gastrodermis. Monné (1959) showed the presence of acid mucopolysaccharide in the eggshell and membranes of 3 species of lungworms, a finding at odds with the present work. Helminths such as *C. lari* that have access to a rich supply of oxygen via the host blood or live in aerobic conditions tend to have little glycogen in their tissues. This is unlike the situation seen in anaerobic or anoxybiotic organisms such as *Ascaris lumbricoides* and *Porrocaecum decipiens* (Barrett, 1981).

Lipids were detected in the cuticle, hypodermis, and muscle cell cytoplasm. This distribution was similar to that noted by Sood and Kalra (1977). The intestine was particularly rich in lipid globules as noted previously by Colam (1971a). Sood and Sehajpal (1978) observed lipids in the esophagus and intestine of *H. contortus* and noted that lipids may well form a major storage product in nematodes. No lipids were detected in the esophagus of *C. lari,* whereas the intestine stained intensely for these substances. The concentration of lipid globules in the intestinal wall of *C. lari* may represent the end-product of digestion in the gut lumen and the syncytium of the gastrodermis. The greater concentration in the anterior region of the intestine suggests that this is where the greatest biochemical activity is occurring. The ovaries were well supplied with lipids, with lesser amounts being detected in the ovum. The eggshell was rich in lipids, a phenomenon previously reported in other nematode species (Rogers, 1962; Seesee, 1977). Lipids were confined to the outer and inner layers of the 3-layered eggshell.

Proteins form the major structural component of *C. lari* as reflected by the general orthochromasia with mercuric bromphenol blue. The cuticle, hypodermis, lateral cords, and muscles, particularly the myofilaments, stained lightly. The intestine and reproductive system were rich in proteins. Many enzymes are proteinaceous in nature. The present results suggest that the anterior intestine is most heavily involved in the digestive process. This observation supports the work of Colam (1971a), but refines our knowledge by showing that enzymatic activity may vary along the length of the intestine. The ovary is a region of intense protein, DNA, and mitochondrial activity resulting in the formation of ova. The eggshells stained quite intensely with mercuric bromphenol blue, probably as a result of their being composed of lipoproteins (Seesee, 1977).

RNA was detected in the intestine; the most intense reaction was in the anterior portion, with less intense reactions in the mid and posterior portions and rectum. The ovaries and ova were also rich in this substance. The hypodermis and hypodermal cords did not stain for RNA, which differs from the results of Sood and Kalra (1977), who noted a reaction in the hypodermis, hypodermal cords, and inner cortical layer of the cuticle of *H. contortus*. Barrett (1981) noted the types of RNA found in nematodes and discussed their importance in protein building. The gut contents of the nematode, host blood containing nucleated erythrocytes and leucocytes, stained intensely for RNA.

Cyathostoma lari is an obligate hematophage, with its "normal" red coloration being due to the presence of host blood in its gut and a red pigment in its pseudocoel. Tests revealed the presence of hemoglobin (Hb) in the intestinal lumen, and in the protein-containing pseudo-

coelomic fluid. No attempt was made to characterize the Hb in the pseudocoel. Colam (1971a) noted Hb in the gut lumen of *C. lari* and as small granules below the brush layer of the gastrodermis after hemolysis. Sood and Sehajpal (1978) noted Hb in the same location in the intestine of *H. contortus,* whereas Sood and Kalra (1977) identified Hb in the cuticle of *H. contortus.* Rose and Kaplan (1972), working on the closely related species *Syngamus trachea,* noted that the Hb from the worm was composed of only 1 component and had a different molecular weight than that extracted from the host blood. This result differs from that of van Grembergen (1954) who showed that the Hb of *Heterakis gallinae* had characteristic alpha and beta absorption bands that were similar to those shown by the Hb from host blood. Hemoglobins in nematodes seem to have a very high affinity for oxygen, even at very low partial pressures. Oxyhemoglobin will, therefore, only dissociate at very low tissue concentrations of oxygen, and it seems improbable that the Hb in an aerobic species, such as *C. lari,* will be used for oxygen transport. The differences noted above in the types of Hb found in nematodes, as well as the function of Hb, are deserving of further investigation.

Mitochondria, indicated by the presence of succinic dehydrogenase, were detected in greatest amounts in the anterior part of the intestine. Lesser amounts were present in the remainder of the digestive tract (except for the wall of the buccal cavity and esophagus where they were absent). The reproductive tract was also positive. These locations all correspond to sites of great biochemical activity. Colam (1971a) showed that digestion and absorption of blood cells occurred in the gastrodermis of *C. lari,* and that numerous multicristate mitochondria were present in specific regions of the tissue. Monné (1959) discussed the mitochondria of developing lungworm ova, and noted that they are numerous both at this stage and in adult worms.

Acid and alkaline phosphatases were widely distributed in the digestive and reproductive tracts, regions where intense biochemical activity might be expected. Again the anterior intestine appeared to be the region where most activity occurred, with a decline of activity in the posterior regions. Both these substances are associated with digestion (Colam, 1971a; Riley, 1973). The presence of acid phosphatase has been used as an indicator of lysosomal activity (Duvé, 1963; Novikoff, 1963) and would be expected to occur in areas where intense biosynthesis is occurring. The role, and distribution, of phosphatases in cestodes is somewhat better understood than in nematodes (Threlfall et al., 1990). Arme and Read (1970) and Mayberry and Tibbitts (1972) suggested that alkaline phosphatase is involved in active transport and/or digestion. Other workers (Sood and Kalra, 1977; Sood and Sehajpal, 1978; Maki and Yanagisawa, 1980) have shown a more general distribution of phosphatases in nematodes than was seen in *C. lari.* In the present work the cuticle, hypodermis, hypodermal cords, and muscles appeared to be free of phosphatases.

Cyathostoma lari is typical of many strongyloids in possessing a limited number of cells (Chitwood and Chitwood, 1974) and a syncytial intestinal wall (Colam, 1971a). The presence of a syncytial intestine, which has a well-developed bacillary (microvillar) layer, may be an adaptation to hematophagy. Differences noted in the amounts of various substances along the length of the intestine suggest differential enzymatic action along its length and are worthy of further study.

It became obvious that marked differences do occur in the presence and distribution of substances in different nematode species. Our knowledge of such differences is at present rudimentary. A more complete understanding of the physiological and biochemical processes occurring in nematodes will be aided by further histochemical studies, including a comparison of male and female worms. A preliminary study of male *C. lari,* which are much rarer and smaller than the females (Burt and Eadie, 1958), revealed chemical substances and distributions similar to those noted above. It is possible that similarities or differences among nematode species might be partially explained by differing habitats of the parasites, stage of development, host phylogeny, and/or host physiological differences.

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Research Note

Experimental Fascioliasis in Llamas

LORA G. RICKARD[1] AND WILLIAM J. FOREYT[2]

[1] College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331 and
[2] Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, Washington 99164

ABSTRACT: Three llamas and 2 domestic sheep were inoculated orally with metacercariae of liver flukes, *Fasciola hepatica.* The prepatent period in llamas and sheep was 8–12 wk. Sizes of fluke eggs passed in feces were similar between llamas and sheep. At necropsy, the percentages of original inoculum recovered from the llamas and sheep were 24% and 22%, respectively. Sizes of flukes recovered from livers were similar between llamas and sheep. The gross appearance of the livers from the llamas varied from slight discoloration with some bile duct thickening to marked fibrosis and scarring. Llama livers were similar histologically. Bile duct hyperplasia, portal fibrosis, and granulomas, often containing degenerated trematode eggs and necrotic debris, were hallmarks of infection. These changes resembled chronic fascioliasis in sheep. The data indicate that llamas, like domestic sheep, have low resistance to liver fluke infection.

KEY WORDS: *Fasciola hepatica,* liver flukes, experimental infection, llama, *Lama glama,* sheep, *Ovis aries.*

Fasciola hepatica is a prevalent and economically important trematode parasite of cattle and sheep in the United States. In endemic areas goats, rabbits, swine, horses, and man may also become infected (Leathers et al., 1982; Soulsby, 1982; Malone, 1986; Wescott and Foreyt, 1986). In the United States, natural infections of *F. hepatica* have been reported in 1 llama in Texas and 1 llama in Oregon (Cornick, 1988; Rickard and Bishop, 1991). The purpose of this study was to determine the prepatent period of *F. hepatica* in llamas, describe the lesions associated with mature infections, and compare them with those in domestic sheep.

Three healthy adult female llamas (*Lama glama*), 5–7 years old, were donated for research purposes because of reproductive or conformational problems. All were maintained on pasture and were supplemented with hay when needed. All pastures used were known fluke-free pastures and none of the animals had any history of liver fluke infections prior to experimental infection. All llamas were clinically normal. Two of the llamas (nos. 2 and 3) were also given larvae of meningeal worm, *Parelaphostrongylus tenuis,* on day 18 of this experiment. Because *P. tenuis* in llamas is confined to the neurologic system (Baumgartner et al., 1985; Krogdahl et al., 1987), it was considered that it would not directly affect the liver fluke infection. Two healthy domestic sheep (*Ovis aries*), 1.5-year-old wethers, were purchased as lambs from a known *F. hepatica*-free area, and were housed on pasture until the start of the experiment when they were moved indoors and housed on concrete.

On day 0, 250 (llama 1) or 500 (all other animals) metacercariae of *F. hepatica* (Baldwin Enterprises, Monmouth, Oregon) were administered to each animal orally either by stomach tube (llamas) or gelatin capsule (sheep). Rectal fecal samples were collected and animals were weighed at approximately 2-wk intervals throughout the trial. Animals were observed daily for signs of clinical parasitism.

Five grams of feces was examined at each sampling period for eggs of *F. hepatica* with a sedimentation technique. For llama 1, the samples were scored as negative or positive, and for the other animals, actual numbers of fluke eggs per gram of feces were determined. A minimum of 20 eggs from each positive sample were measured using a microscope equipped with an ocular micrometer.

On day 157 postinfection, llama 1 was euthanized for reasons unrelated to parasitism. On